FEBS Letters 408 (1997) 206–210 FEBS 18572

Transmembrane topology of the Rieske Fe/S protein of the cytochrome b_6/f complex from spinach chloroplasts

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Received 1 April 1997

Abstract The topology of the Rieske protein of the cytochrome b_6/f complex in thylakoids from spinach chloroplasts was examined by protease protection experiments as well as polypeptide extraction assays using solutions of chaotropic salts or alkaline pH. While neither thermolysin nor trypsin cleave any of the Rieske protein when added to the stromal side of the thylakoid membrane, proteinase K is capable of removing approximately four residues from its NH_2-terminus. The protein is resistant to membrane extraction by 0.1 M Na_2CO_3 or 2 M NaBr but is quantitatively released by 0.1 M NaOH. Treatment of thylakoids with 2 M NaSCN leads to extraction of variable amounts of the protein, depending on the presence or absence of sucrose in the medium which apparently stabilizes the cytochrome complex. From these results we conclude that the Rieske protein is an integral component of the cytochrome complex which spans the thylakoid membrane with a single hydrophobic segment and is anchored predominantly by electrostatic interactions.

Key words: Rieske Fe/S protein; Cytochrome b_6/f complex; Thylakoid membrane; Chloroplast; Photosynthesis; Protein topology

1. Introduction

The cytochrome b_6/f complex of chloroplasts, the plastoquinol/plastocyanin-oxidoreductase in the photosynthetic electron transport chain, is an integral multisubunit assembly that is found both in stacked and unstacked regions of the thylakoid membrane system (for a recent review see [1]). It is composed of at least seven polypeptide species of which four, notably the cytochromes *f* (gene: *pet*A) and b_6 (*pet*B), the Rieske Fe/S protein (*pet*C), and subunit IV (*pet*D), are apparently sufficient for oxidoreductase activity [2]. The function of the three additional subunits of low molecular mass, subunits PetG, PetL, and PetM [3–8], is as yet unknown, except that absence of PetG or PetL causes a general instability of the complex [8,9].

Three of the four core subunits of the cytochrome complex are integral membrane proteins which span the membrane with one (cytochrome *f*), three (subunit IV), and four (cytochrome b_6) transmembrane helices, respectively [10–13]. In contrast, the topology of the Rieske protein is a matter of considerable dispute (e.g., [14,15]). Close to its NH_2-terminus, the Rieske protein houses a stretch of 25 hydrophobic residues which might either serve to attach the protein from the lumenal side to the membrane [15,16] or to anchor it within the membrane with one or two transmembrane spans [17,18]. The bulk of the protein including the COOH-terminus is located in the thylakoid lumen. It carries characteristically spaced, highly conserved cysteine and histidine residues [17] which apparently provide the ligands for the prosthetic [2Fe–2S] group involved in the electron transfer from plastoquinol to cytochrome *f* [19]. In higher plants, the Rieske protein is encoded in the nucleus and synthesized in the cytosol as a precursor molecule with an NH_2-terminal transit peptide [17,20,21]. This transit peptide is required for transport across the envelope membranes but not for the subsequent thylakoid targeting of the protein [22]. Thylakoid transport is instead mediated by signals in the mature protein which include the NH_2-terminal hydrophobic segment [23].

In the course of protein transport experiments which have been performed to characterize the translocation pathway for the Rieske protein across the thylakoid membrane, we obtained data suggesting that the NH_2-terminus of the protein is exposed on the stromal face of the thylakoid membrane (I. Karnauchov, S. Clausmeyer, R.G. Herrmann, and R.B. Kösgen, manuscript in preparation). In order to examine whether this finding reflects the in vivo situation or whether it is a consequence of incomplete membrane transfer and/or assembly taking place in the *in organello* system, comparison to the native protein is required. We have therefore studied the topology of the Rieske protein by analyzing thylakoids that had either been treated with proteases or extracted with solution of chaotropic salts or alkaline pH. The results were compared with those obtained for other subunits of the cytochrome b_6/f complex.

2. Methods

2.1. Materials

Spinach (*Spinacia oleracea*) was purchased from the local market and kept over night at 4°C before isolating chloroplasts and thylakoids. Trypsin, trypsin inhibitor, thermolysin, and alkaline phosphatase-coupled secondary antibody were obtained from Sigma (Munich, Germany), and proteinase K and phenylmethylsulfonylfluoride (PMSF) were from Merck (Darmstadt, Germany). Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were purchased from Serva (Heidelberg, Germany).

2.2. Isolation of thylakoids from spinach chloroplasts

Intact chloroplasts were prepared from spinach leaves and purified on Percoll gradients following the protocol of Bartlett et al. [24]. Chloroplasts were lyzed by osmotic shock for 5 min on ice at 0.5 mg chlorophyll/ml in HM buffer (10 mM HEPES–KOH, pH 8.0, 5 mM $MgCl_2$). Thylakoids were collected by centrifugation for 5 min at 20 000 × *g*, washed twice in HM buffer, and finally resuspended at 0.5 mg chlorophyll/ml in either HM or HS buffer (10 mM HEPES–KOH, pH 8.0, 0.1 M sucrose), depending on the subsequent experiment.

2.3. Protease treatment of membranes

Freshly isolated thylakoids resuspended in HS buffer were incu-

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PII S0014-5793(97)00427-4

bated for 30 min with either 100 μg/ml thermolysin, 10 μg/ml trypsin or 15 μg/ml proteinase K. Proteolysis was stopped by supplementing the assays with 20 mM EDTA (thermolysin), 50 μg/ml soybean trypsin inhibitor (trypsin), or 2 mM PMSF (proteinase K). Thylakoids were re-isolated by centrifugation for 5 min at 20000×*g*, washed once in HS buffer containing the respective protease inhibitor and subjected to SDS–polyacrylamide gel electrophoresis [25]. Transfer of thylakoid proteins to nitrocellulose membranes and Western analysis followed the protocols of Towbin et al. [26] and Blake et al. [27], respectively.

2.4. Polypeptide extraction from thylakoids

Freshly isolated thylakoids were resuspended at 0.5 mg chlorophyll/ml in 2 M NaBr, 2 M NaSCN, 0.1 M Na_2CO_3, or 0.1 M NaOH dissolved in either HM or HS buffer. After incubation on ice for 30 min, the assays were diluted with two volumes of the respective buffer and centrifuged at 20000×*g* for 10 min. Proteins were solubilized in SDS sample buffer either directly (pellet fraction) or after precipitation in 80% acetone (supernatant fraction), separated by SDS–polyacrylamide gel electrophoresis [25] and subsequently subjected to Western analysis [26,27].

2.5. Miscellaneous

Western analysis was performed according to Blake et al. [27] with 50 μg/ml BCIP and 100 μg/ml NBT at 22°C until appropriate color development. All other methods followed the protocols of Sambrook et al. [28].

3. Results

3.1. The Rieske protein is accessible to protease from the stromal side of the thylakoid membrane

Integral membrane proteins generally protrude into the surrounding hydrophilic matrix and are therefore accessible to limited degradation in the presence of proteolytic enzymes. To study the topology of the Rieske protein of the cytochrome b_6/f complex, we have determined its sensitivity to trypsin, thermolysin, or proteinase K that were added to isolated thylakoids. The reactions were carried out in parallel at 0°C and 20°C, and the fate of the Rieske protein was traced in Western experiments using polyclonal, monospecific antisera.

At 20°C, the Rieske protein is cleaved by proteinase K to a polypeptide with moderately increased electrophoretic mobility under denaturating conditions (Fig. 1). From this mobility it was deduced that approximately four amino acid residues had been removed by the enzyme. This corresponds well with the presence of potential target residues at positions 4 (isoleucine) and 6 (alanine) of the polypeptide chain [17] and suggests a bitopic membrane topology of the protein with the NH_2-terminus facing the stroma. On the other hand, neither thermolysin nor trypsin are capable to degrade the Rieske protein to any detectable degree (Fig. 1). This substantiates that only a small portion of the assembled protein faces the stroma, because potential target sites for thermolysin or trypsin are lacking from the first 11 residues of the protein.

Cleavage of the Rieske protein by proteinase K was not caused by disruption of thylakoids and subsequent proteolysis of the lumenal domain, because in lysed thylakoids the protein is almost completely degraded by the enzyme (data not shown). Further proof for the integrity of the thylakoids is provided by the 33 kDa protein of the oxygen-evolving system, a peripheral membrane component of the thylakoid lumen. This protein is unaffected by proteinase K under the chosen assay conditions (Fig. 1) demonstrating that the thylakoids remained intact in the course of the experiment. Therefore, cleavage of the Rieske protein must have occurred from the stromal side. We conclude from this that the protein is embedded with a single transmembrane segment in the lipid bilayer and faces the stroma with few NH_2-terminal residues.

Even at 0°C, the Rieske protein is accessible to proteinase K, though to a much lesser extent (Fig. 1). The majority of the protein remains unaffected under these conditions and only a minute fraction is cleaved by the enzyme, as judged from the appearance of a slight smear on denaturing protein gels (Fig. 1). This finding may indicate that the NH_2-terminus of the Rieske protein is not permanently exposed at the stromal face of the membrane but for most of the time is shielded and requires a certain membrane fluidity to protrude temporarily.

Interestingly, cytochrome *f* behaves comparably to the Rieske protein in such assays. After proteinase K treatment of thylakoids at 20°C, cytochrome *f* shows a moderate increase in its electrophoretic mobility which in this instance corresponds to the removal of approximately 10 residues (Fig. 1), in agreement with results obtained for pea chloroplasts [10]. Cleavage of cytochrome *f* takes place with significantly lower efficiency at 0°C, and the protein is as well resistant to both thermolysin and trypsin at either of the two temperatures assayed (Fig. 1).

3.2. The Rieske protein is anchored in the thylakoid membrane by electrostatic interactions

In order to obtain information of how the Rieske protein is anchored in the membrane, we have treated isolated thylakoids with solutions of chaotropic salts or alkaline pH, and assayed the resulting fractions in Western experiments. It turned out that the Rieske protein remains membrane-bound in the presence of 0.1 M Na_2CO_3 (pH 11.4) in agreement with the transmembrane topology of the protein, but that it is almost quantitatively released by 0.1 M NaOH (Fig. 2). Thus, at pH 13.0 the majority of the interactions that are required to anchor the protein in the thylakoid membrane is destroyed suggesting that they are predominantly of electrostatic nature [14].

Hydrophobic interactions participate as well in anchoring the Rieske protein to the thylakoid membrane but apparently to a minor extent only. If thylakoids are treated with solutions of chaotropic salts such as 2 M NaSCN, the majority of the protein persists in the membrane and only a small fraction is released (Fig. 2). Treatment with 2 M NaBr does not even release any of the protein (Fig. 2) which suggests that extraction of the Rieske protein by chaotropic salts can only take place under conditions in which the integrity of the thylakoids is destroyed. This assumption is supported by the results for the 33 kDa protein of the oxygen-evolving system which show that hydrophilic proteins of the thylakoids lumen can be quantitatively released by NaSCN but are not accessible to extraction by NaBr or Na_2CO_3 (Fig. 2).

None of the other components of high molecular mass of the cytochrome b_6/f complex displays a behavior comparable to that of the Rieske protein in this type of analysis. Both cytochrome *f* and subunit IV are resistant against all extraction procedures suggesting that they both are anchored in the membrane by strong electrostatic and hydrophobic forces (Fig. 2). On the other hand, cytochrome b_6 is almost quantitatively released by either NaOH, Na_2CO_3, or NaSCN (Fig. 2). Even NaBr is capable to extract most of this protein indicating That cytochrome b_6 exerts only weak interactions within the lipid bilayer, in spite of the fact that it possesses

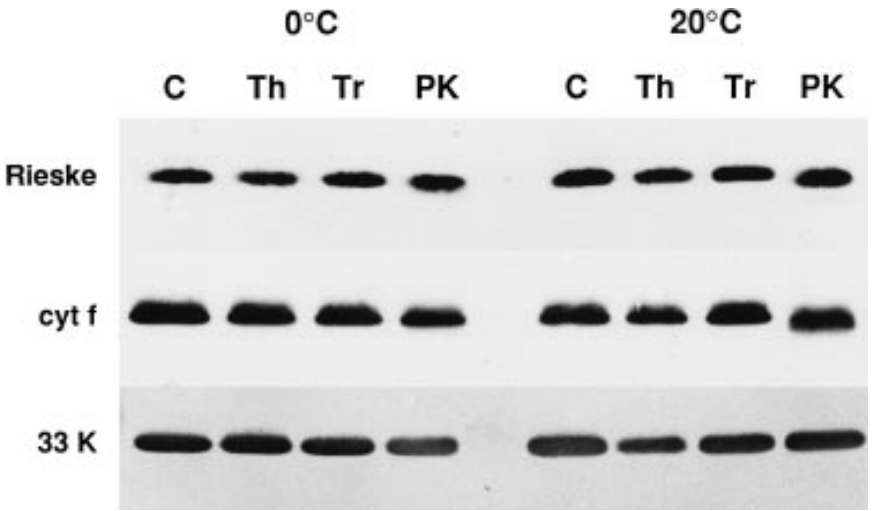

Fig. 1. Effect of protease treatment of thylakoids on the Rieske protein, cytochrome *f* and the 33 kDa subunit of the oxygen-evolving system from spinach. Spinach thylakoids obtained by osmotic lysis of intact isolated chloroplasts were resuspended at 0.5 mg chlorophyll/ml in HS buffer (10 mM HEPES–KOH, pH 8.0, 0.1 M sucrose) containing either 100 μg/ml thermolysin (lanes Th), 10 μg/ml trypsin (lanes Tr), 15 μg/ml proteinase K (lanes PK) or no protease (lanes C). After incubation for 30 min at the temperature indicated, proteolysis was stopped by either 20 mM EDTA (thermolysin), 50 μg/ml soybean trypsin inhibitor (trypsin), or 2 mM PMSF (proteinase K). Thylakoids were collected, solubilized in SDS sample buffer and heat-denatured for 2 min at 100°C. Stoichiometric amounts of each assay, corresponding to 18 μg chlorophyll, were separated on 10–17.5% SDS–polyacrylamide gradient gels and analyzed in Western experiments using polyclonal antisera raised against the proteins indicated.

four transmembrane segments [13]. This observation stands in contradiction to results described for cytochrome b_6 from *Chlamydomonas reinhardtii* which showed strong membrane persistence of the protein in the presence of chaotropic salts or alkaline pH [15]. The reason for this difference is unknown.

3.3. *The Rieske protein is stabilized in the thylakoid membrane in the presence of sucrose*

As cytochrome b_6, the Rieske protein from *Chlamydomonas* behaves differently from its spinach homolog in the polypeptide extraction assays as well. In the alga, the protein is almost quantitatively extracted from thylakoid membranes by 2 M KSCN, and even 1.5 M NaI releases more than 50% of it into the supernatant [15]. These results led the authors to conclude that the Rieske protein is an extrinsic protein which is only attached from the lumenal side to the thylakoid membrane but does not assume a transmembrane topology [15]. Although we cannot rule this out, it appears unlikely that

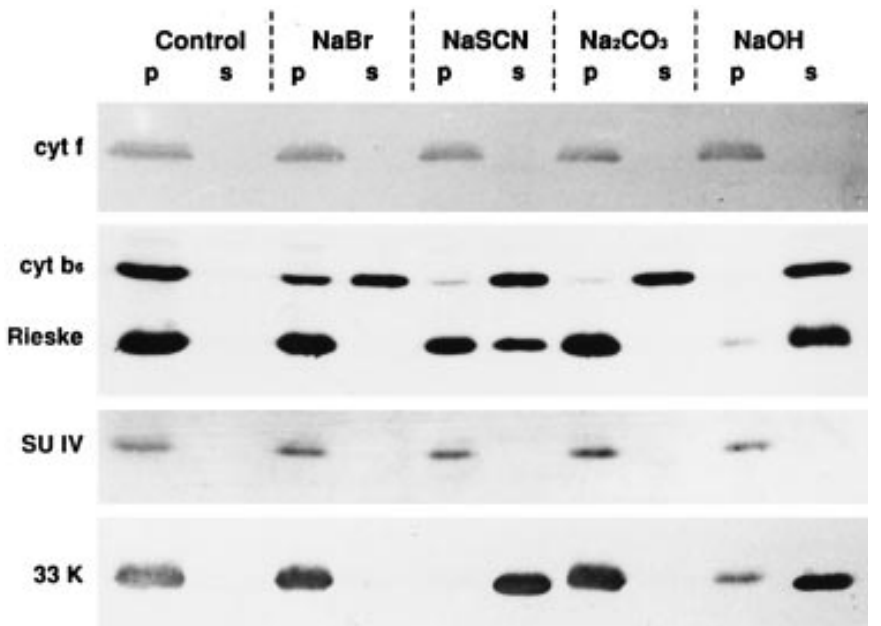

Fig. 2. Extraction of subunits of the cytochrome b_6/f complex by solutions of chaotropic salts or alkaline pH. Isolated spinach thylakoids were resuspended at 0.5 mg chlorophyll/ml in HS buffer containing either 2 M NaBr, 2 M NaSCN, 0.1 M Na_2CO_3, 0.1 M NaOH, or no additive. After incubation for 30 min on ice, the assays were separated into membrane fraction (*p*) and supernatants (*s*) and analyzed as described in the legend to Fig. 1.

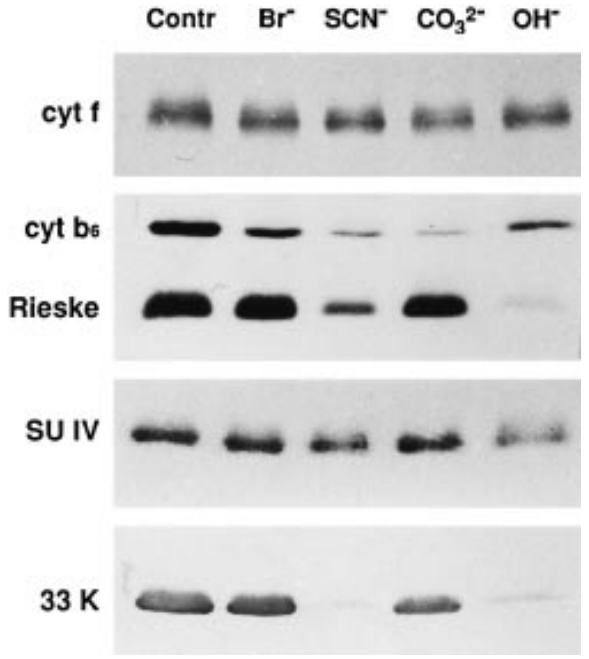

Fig. 3. Effect of membrane stabilization on the extraction of subunits of the cytochrome b_6/f complex from isolated thylakoids. Isolated spinach thylakoids were resuspended at 0.5 mg chlorophyll/ml in HM buffer (10 mM HEPES–KOH, pH 8.0, 5 mM $MgCl_2$) containing either 2 M NaBr (Br^-) 2 M NaSCN (SCN^-), 0.1 M Na_2CO_3 (CO_3^{2-}), 0.1 M NaOH (OH^-), or no additive (Contr). Protein extraction was performed as in the experiment shown in Fig. 2, except that only membrane fractions were analyzed.

the Rieske protein from *Chlamydomonas* possesses indeed a different membrane topology than its higher plant homolog. Instead, the conflicting results might be a consequence of different methods in the isolation and treatment of thylakoids. While we have tried to preserve an almost native structure of the isolated spinach thylakoids, thylakoids prepared from *Chlamydomonas* were stored at −80°C, treated with repeated freeze/thaw cycles during polypeptide extraction and lacked membrane stabilization by sucrose [15].

In order to examplarily study the possible influence of such differences, the experiments presented in Fig. 2 were repeated also with spinach thylakoids that were kept in buffer lacking sucrose as a stabilizing agent. Indeed, the Rieske protein is more sensitive to extraction by NaSCN under these conditions, although it is still not quantitatively released (Fig. 3). Inspection of the Western signals suggests that in the absence of sucrose up to 90% of the protein can be extracted from the membrane (Fig. 3, and data not shown) but only approximately 30% from thylakoid stabilized by sucrose (Fig. 2, and data not shown). On the other hand, the presence of sucrose has no influence on the extraction of the Rieske protein by NaOH (Fig. 3) which suggests that such stabilization can be effective only within a limited range.

Lack of membrane stabilization does not explain the contradictory results for cytochrome b_6 in spinach and *Chlamydomonas*, because in all instances the spinach protein is extracted by NaSCN, NaBr, Na_2CO_3, or NaOH to approximately the same degree, independent of the presence or absence of sucrose in the thylakoid preparations (Figs. 2 and 3).

4. Discussion

It was the principal goal of this study to resolve the long-lasting ambiguity found in current literature concerning the topology of the Rieske protein from the cytochrome b_6/f complex of chloroplasts. Our data demonstrate unequivocally that the protein is an integral component of the thylakoid membrane which carries a single transmembrane segment and faces the stroma with few NH_2-terminal residues.

This statement rests on two observations. When added to isolated thylakoids, proteinase K is capable to cleave the Rieske protein to a product lacking approximately four amino acid residues (Fig. 1). Since the COOH-terminus of the protein is located in the thylakoid lumen, cleavage must have taken place at the NH_2-terminal end of the Rieske protein. At 0°C, cleavage by proteinase K is barely detectable and, similarly, also cytochrome *f* is cleaved by proteinase K at 20°C but not at 0°C. This suggests that at low temperature the entire cytochrome complex might be protected from proteolytic attack indicating that it is only temporarily exposed to the stromal space under conditions of sufficient membrane fluidity.

Neither thermolysin nor trypsin are capable to degrade any of the Rieske protein from the stromal face of the thylakoid membrane, not even after pretreatment of the thylakoids with 2 M NaBr (data not shown). This indicates that probably phospholipids rather than proteinaceous components of the membrane are responsible for this protection.

Second indication for a transmembrane topology of the Rieske protein is its persistence in the thylakoid membrane even after washing with 0.1 M Na_2CO_3 (Figs. 2 and 3). On the other hand, chaotropic salts extract some of the protein under conditions in which the thylakoid vesicles are destroyed. This suggests that the Rieske protein is anchored predominantly by electrostatic interactions rather than hydrophobic interactions. In the presence of sucrose, the amount of Rieske protein released by the chaotropic agent is reduced. Such stabilization of protein complexes must therefore be considered as an important factor which can to a large extent influence the membrane persistence of proteins in this type of study.

Interestingly, also in the cytochrome b/c_1 complex of mitochondria, the ubiquinol/cytochrome *c*-oxidoreductase in the respiratory electron transport chain, the topology of the Rieske protein has not yet finally been settled. Like its plastidic counterpart, the mitochondrial Rieske protein carries a hydrophobic segment close to its NH_2-terminus, and both a membrane-attached and a transmembrane topology have been proposed [29–33]. The close functional and phylogenetic relationship of the two cytochrome complexes suggests that the mitochondrial Rieske protein possesses a bitopic transmembrane topology as well but proof is still lacking in this instance. It should be noted that even the X-ray structure of the crystallized cytochrome b/c_1 complex from bovine mitochondria which was recently determined at 3.4 Å resolution [34] does not provide unambiguous information about the topology of the Rieske protein.

It is remarkable that the four subunits of the cytochrome b_6/f complex that have been analyzed in this study show major differences in their membrane persistence in the presence of chaotropic salts and solutions of alkaline pH, in spite of the fact that they are all integral components of the thylakoid

membrane. Unexpectedly, just the protein with the highest number of transmembrane spans, cytochrome b_6, is most easily released by such treatments, whereas the bitopic cytochrome *f* is strongly anchored within the membrane with its single transmembrane segment. These findings show clearly that studies concerning the topology of membrane proteins which rely solely on this type on analysis have to be interpreted with caution.

Acknowledgements: This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 184).

References

[1] W.A. Cramer, G.M. Soriano, M. Ponomarev, D. Huang, H. Zhang, S.E. Martinez, J.L. Smith, Annu. Rev. Plant Physiol. Plant Mol. Biol. 47 (1996) 477–508.
[2] E. Hurt, G. Hauska, Eur. J. Biochem. 117 (1981) 591–599.
[3] J. Haley, L. Bogorad, Proc. Natl. Acad. Sci. USA 86 (1989) 1534–1538.
[4] C.L. Schmidt, R. Malkin, Photosyn. Res. 38 (1993) 73–81.
[5] Y. Pierre, C. Breyton, D. Kramer, J.-L. Popot, J. Biol. Chem. 270 (1995) 29342–29349.
[6] C. De Vitry, C. Breyton, Y. Pierre, J.-L. Popot, J. Biol. Chem. 271 (1996) 10667–10671.
[7] S.L. Ketchner, R. Malkin, Biochim. Biophys. Acta, Gene Struct. Exp. 1273 (1996) 195–197.
[8] Y. Takahashi, M. Rahire, C. Breyton, J.-L. Popot, P. Joliot, J.-D. Rochaix, EMBO J. 15 (1996) 3498–3506.
[9] D.A. Berthold, C.L. Schmidt, R. Malkin, J. Biol. Chem. 270 (1995) 29293–29298.
[10] D.L. Willey, A.D. Auffret, J.C. Gray, Cell 36 (1984) 555–562.
[11] W.A. Cramer, W.R. Widger, R.G. Herrmann, A. Trebst, Trends Biochem. Sci. 10 (1985) 125–129.
[12] M.P. Doyle, L.-B. Li, L. Yu, C.-A. Yu, J. Biol. Chem. 264 (1989) 1387–1392.
[13] A. Szczepaniak, W.A. Cramer, J. Biol. Chem. 265 (1990) 17720–17726.
[14] A. Szczepaniak, D. Huang, T.W. Keenan, W.A. Cramer, EMBO J. 10 (1991) 2757–2764.
[15] C. Breyton, C. de Vitry, J.-L. Popot, J. Biol. Chem. 269 (1994) 7597–7602.
[16] A. Szczepaniak, K. Frank, J. Rybka, Z. Naturforsch. 50c (1995) 535–542.
[17] J. Steppuhn, C. Rother, J. Hermans, T. Jansen, J. Salnikow, G. Hauska, R.G. Herrmann, Mol. Gen. Genet. 210 (1987) 171–177.
[18] D.L. Willey, J.C. Gray, Protosyn. Res. 17 (1988) 125–144.
[19] E. Davidson, T. Ohnishi, Atta-Asafo-Adjei, F. Daldal, Biochemistry 31 (1992) 3342-3351.
[20] F. Madueno, J.A. Napier, F.J. Cejudo, J. Gray, Plant Mol. Biol. 20 (1992) 289–299.
[21] A.H. Salter, B.J. Newman, J.A. Napier, J.C. Gray, Plant Mol. Biol. 20 (1992) 569–574.
[22] D. Bartling, S. Clausmeyer, R. Oelmüller, R.G. Herrmann, Bot. Mag. Tokyo 2, Special Issue 2 (1990) 119-144.
[23] F. Madueno, S.A. Bradshaw, J. Gray, J. Biol. Chem. 269 (1994) 17458–17463.
[24] S.G. Bartlett, A.R. Grossman, N.-H. Chua, in: M. Edelman, R.B. Hallick, N.-H. Chua (Eds.), Methods in Chloroplast Molecular Biology, Vol. 86, 1982, pp. 1081-1091.
[25] U.K. Laemmli, Nature 227 (1970) 680–685.
[26] H. Towbin, T. Staehelin, J. Gordon, Proc. Natl. Acad. Sci. USA 76 (1979) 4350–4354.
[27] M.S. Blake, K.H. Johnston, G.T. Russel-Jones, E.C. Gotschlich, Anal. Biochem. 136 (1984) 175–179.
[28] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning. a Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[29] U. Harnisch, H. Weiss, W. Seebald, Eur. J. Biochem. 149 (1985) 95–99.
[30] F.-U. Hartl, B. Schmist, E. Wachter, H. Weiss, W. Neupert, Cell 47 (1986) 939–951.
[31] H. Schägger, W. Borchart, W. Machleidt, T.A. Link, G. von Jagow, FEBS Lett. 219 (1987) 161–168.
[32] T. Cocco, M. Lorusso, A.M. Sardanelli, M. Minuto, S. Ronchi, G. Tedeschi, S. Papa, Eur. J. Biochem. 195 (1991) 731–734.
[33] D. Gonzalez-Halphen, M. Vazquez-Acevedo, B. Garcia-Ponce, J. Biol. Chem. 266 (1991) 3870–3876.
[34] C.A. Yu, J.Z. Xia, A.M. Kachurin, L. Yu, D. Xia, H. Kim, J. Deisenhofer, Biochim. Biophys. Acta Bioenerg. 1275 (1996) 47–53.